



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Nicolaides *et al.*

Serial No.: 09/558,149

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Examiner: R. Shukla

For: METHODS FOR GENERATING HYPERMUTABLE ORGANISMS

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Dated
10/21/02

Assistant Commissioner for Patents
Washington D.C. 20231

Declaration under 37 CFR 1.131

I, J. Bradford Kline, hereby state the following:

1. I earned my doctorate from the University of Miami, Department of Microbiology and Immunology in 1996 in the field of Molecular Microbial Pathogenesis.
2. I am thoroughly familiar with the field of transgenic animals, and I am a Senior Scientist working on transgenic animals for Morphotek Inc. in the Mammalian Genetics Group.
3. I have been employed by Morphotek since May 2001, and have been working under the direction of Drs. Nicholas C. Nicolaides, Luigi Grasso and Philip M. Sass on transgenic animals expressing dominant negative alleles of mismatch repair genes.
4. I have read and understand the disclosure regarding the generation of transgenic animals in U.S. Patent No. 6,146,894 to Nicolaides *et al.* ("the '894 patent").
5. Following the guidelines of the '894 patent, we generated transgenic mice as follows:

Transgene Fragment Generation

A plasmid containing a sequence for a PMS-134 truncation mutant driven by a pEF promoter was constructed and a fragment of the plasmid containing the promoter and PMS-134 sequence was restriction digested and purified by agarose gel electrophoresis and subsequent electroelution in TBE buffer. The fragment was ethanol precipitated, washed twice with

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70% ethanol, and the DNA was dissolved in injection buffer at a final concentration of 5 µg/ml.

Microinjection of the Transgene

Yale University's Transgenic Mouse Service was used for the microinjection of 250 pronuclear embryos of C57BL/6JxSJL/J F2 mice with the fragment containing the PMS-134 sequence. Yale University's Transgenic Mouse Service uses routine techniques known in the art for preparing transgenic animals. That is, the fragment was microinjected and the embryos were transferred to the uteri of six pseudopregnant foster mothers and brought to term. Tail biopsies of weanling mice were extracted 4 weeks after birth for genetic analysis.

Genomic Screening of Founder Mice for the Transgene

Tail clippings of potential founder mice were used as a source for genomic DNA purification using the Dneasy DNA purification kit (Qiagen). A 5-10 ng sample of DNA from each genomic sample was used as template for PCR amplification of the PMS-134 fragment (See Figure 1). The oligonucleotide primers used for amplification were homologous to the transgene fragment immediately upstream and downstream of the PMS-134 sequence. A PMS-134 transfected cell line was used as a positive control, while wild-type mouse DNA was used as a negative control. 5 µl of the PCR reactions were analyzed by agarose gel electrophoresis for the presence of the PMS-134 sequence. The results are shown in Figure 1 wherein it is shown that 11 animals contained the PMS-134 sequence.

Southern Analysis of the Transgenic Mice

5 µg of genomic DNA from PCR-positive transgenic founder mice was digested overnight with a restriction enzyme. The samples were electrophoresed on a 1% agarose gel and transferred to a PVDF membrane. The blot was probed with a PCR-generated, radiolabeled PMS-134 fragment. After washing, the blot was analyzed by autoradiography. A 2.2 kb band is indicative of head to tail concatemer integration of the transgene. The results are shown in Figure 2, wherein it is shown that 5 animals (673, 683, 685, 704 and 708) contained a head to tail concatemer integration of the PMS-134 transgene.

PCR Screening for Germline Transmission of the Transgene

Potential transgenic founder mice were backcrossed to wild-type C57/B6 mice to determine if the transgene is transmitted through the germline. Tail clippings of pup litters were used for genomic DNA isolation. PCR was performed using primers upstream of the PMS-134 sequence, and internal to the PMS-134 sequence. A positive result yields a 164 bp DNA fragment. The results are shown in Figure 3 which shows that pups 685.2 and 685.3 inherited the transgene from the founder, 685.

A PMS-134- transfected cell line was used as a positive control, while wild-type mouse DNA (C57/B6) was used as a negative control.

mRNA Tissue Expression of the Transgene in Transgenic Mice

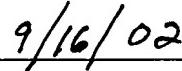
To confirm expression of the transgene, a hemizygous (685.3) and wild-type (C57/B6) mouse were sacrificed and their major organs were harvested. 10 mg of kidney, lung and liver were used for RT-PCR using the Express-Direct kit (Pierce). The RT-PCR samples were run on an agarose gel. The results are shown in Figure 4, which shows transgene expression in the kidney, liver and lungs of the 685.3 mouse, but not in the control mouse. Lanes showing the results or RT-PCR in the absence of reverse transcriptase show that the amplification of the transgene is not from any DNA contamination.

6. All statements made herein of my own knowledge are true, and all statements made herein on information and belief are believed to be true.

7. I hereby acknowledge that willful false statements and the like are punishable by fine or imprisonment, or both under 18 U.S.C. §1001, and may jeopardize the validity of the application or any patent issuing thereon.



J. Bradford Kline



Date